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(54) Title: CONDITIONALLY IMMORTALIZED GERM CELL LINES

(57) Abstract

The invention relates to immortalized and conditionally immortalized germ cell lines, and in particular to immortalized and conditionally immortalized testicular cell lines. Seminiferous-tubule-like structures can be produced *in vitro* using the immortalized cell lines of the present invention. Methods of producing such cell lines are also provided as well as methods for the *in vitro* production of proteins expressed by these cell lines. In a further aspect, the present invention provides methods of controlling the proliferation and differentiation of immortalized germ cells for a variety of purposes, including *in vitro* fertilization and the production of transgenic mice.

CONDITIONALLY IMMORTALIZED GERM CELL LINES

BACKGROUND OF THE INVENTION

        The present invention generally relates to
10 immortalized and conditionally immortalized cell lines,
each associated with spermatogenesis, and uses for such
cell lines.

        Mature sperm cells in animals originate from
germ cells that have undergone a process referred to as
15 spermatogenesis. This process takes place within a
specialized microenvironment of the seminiferous or
sperm-producing tubule located in the testis.

        In maturing to sperm cells, germ cells progress
through several stages of differentiation during this
20 process. For example, mouse germ cells proceed through
twelve stages of meiotic differentiation, including
primitive type A, type A and type B spermatogonia,
preleptotene, leptotene, zygotene and pachytene
spermatocytes (generally referred to as primary
25 spermatocytes), secondary spermatocytes and round
spermatids. Further differentiation to mature sperm
cells is believed to take place during the journey from
the seminiferous tubule to the epididymis in the in vivo
environment. Little is known, however, about the factors
30 and conditions that regulate the differentiation of germ
cells throughout the spermatogenesis process.

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It is known that germ cells are influenced by
various somatic cells within the specialized
microenvironment. The somatic cells that influence germ
cells during spermatogenesis are referred to as Sertoli,
5 peritubular and Leydig cells.

Sertoli cells are the only somatic cells
located within the lumen of the seminiferous tubule.
They are responsible for providing all nutrients and
protein factors needed for germ cell differentiation.
10 Such protein factors include, for example, androgen
binding protein, transferrin, ceruloplasmin, clusterin,
testibumin, plasminogen activator, α-2 macroglobulin,
inhibins, growth factors, type IV collagen, laminin and
others. Sertoli cells respond to the hormone FSH.

15   Myeloid peritubular cells constitute the walls
of the seminiferous tubule and are mostly responsible for
secreting extracellular matrix molecules. Such cells
have been found to secrete fibronectin and types I and IV
collagen. Peritubular cells act cooperatively with
20 Sertoli cells in the formation and deposition of the
extracellular matrix molecules, but the extent of such
interaction is not well understood.

Leydig cells are the single most important
steroidogenic cells in the testis. They metabolize
25 steroids and are believed to be the principal sites of
testosterone production in the testis. Sertoli cells
also metabolize certain testicular steroids but to a
lesser extent than Leydig cells. Leydig cells respond to
hormonal signals, such as LH and hCG, by activating the
30 steroid pathway which leads to the production of
testosterone and other steroids. Such steroids have been
implicated in the process of meiosis and differentiation
of germ cells. However, whether these effects are

exerted directly on the germ cells or indirectly through their effects on the somatic cells is not clear.

Although it is known that somatic cells, extracellular matrix molecules, hormones and steroids
5 influence the differentiation of germ cells, the exact nature of these interactions and of the molecules involved are not known. It appears that spermatogenesis results from the control and regulation of complex and diverse cellular interactions and communications.

10   An understanding of the ability of germ cells to switch from mitotic proliferation to terminal differentiation would be helpful for the understanding and treatment of cancer since tumors are known for their continued cell proliferation. In addition, the study of
15 germ cells in vitro would enable the study, diagnosis and treatment of infertility, and would be useful in evaluating potential gene therapy strategies to correct genetic defects.

Germ cells that have differentiated to mature
20 sperm under controlled conditions would be useful for in vitro fertilization or to produce transgenic animals, such as transgenic mice, by in vitro fertilization.

Thus, a need exists for both immortalized or permanent cell lines, and conditionally immortalized cell
25 lines capable of switching between proliferation and differentiation under appropriate conditions. Such cell lines can be used as in vitro models of the somatic and germ cell lines to study spermatogenesis and for in vitro fertilization as well as other purposes. Attempts at
30 long term cultures of primary somatic cells and mixed primary somatic and germ cells have been largely unsuccessful. Such attempts have generally involved the in vitro cultures of mixed cell types that seldom

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exceeded fifteen days. To date, no permanent germ cell
lines have been established. The present invention
satisfies the need for such cell lines and provides
related advantages as well.

5   SUMMARY OF THE INVENTION

        The present invention provides a set of germ
cell lines which includes both immortalized germ cell
lines, and conditionally immortalized germ cell lines.
More particularly, the invention provides a set of murine
10   testicular cell lines which includes both immortalized
and conditionally immortalized murine testicular germ
cell lines. These murine germ cell lines are
immortalized or conditionally immortalized at various
stages of sperm cell differentiation. The immortalized
15   germ cell lines are permanent cell lines which do not
proceed through meoisis _in vitro_, whereas the
conditionally immortalized cell lines are capable of
switching between proliferation and differentiation under
appropriate conditions.

20   The invention further relates to a method of
controlling the proliferation or differentiation of
immortalized germ cell lines which contain a gene
encoding for an immortalizing molecule by activating or
deactivating the expression of the immortalizing
25   molecule.   Such an immortalized germ cell could be
induced to differentiate into a mature sperm cell _in
vitro_, which in turn can be used for _in vitro_
fertilization.

        In another aspect, the invention also relates
30   to the _in vitro_ production of proteins, such as a
testicular isoform of cytochrome c and LDH-C, which are
expressed by certain of these immortalized or
conditionally immortalized germ cell lines.

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In a further aspect, the present invention
relates to immortalized somatic cell lines derived from
mammalian testis capable of being cultured in vitro for
at least one month or longer. Such somatic cell lines
5 include Sertoli, myeloid peritubular and Leydig cell
lines.

The immortalized somatic cell lines can be
combined with the immortalized germ cell line to produce
seminiferous tubule-like structures in vitro. The
10 structures are useful as in vitro models for the study of
spermatogenesis and cell-cell and cell-matrix interaction
phenomena and to identify molecules involved in the
process of tissue morphogenesis. The structures can be
detected by labeling at least one cell line with a
15 detectable marker, such as a fluorescent marker.

In another aspect of the present invention, the
immortalized germ cell lines can be used to produce
transgenic animals, such as transgenic mice.

BRIEF DESCRIPTION OF THE DRAWINGS

20   Figure 1 shows an immunocytochemical analysis
of germ cell lines GC-2spd(ts) and GC-3spd(ts) grown at
37°C (Figure 1A), and grown at 32°C (Figure 1B).

Figure 2A shows periodic acid-Schiff staining
of the acrosomal granule in GC-2spd(ts) round spermatids;
25 Figure 2B shows immunocytochemical staining of GC-
2spd(ts) of the acrosomal granule in GC-2spd(ts) round
spermatids with monoclonal antibody HS-63. Figure 2C
shows an electron micrograph of the acrosomal granule.

Figure 3 shows flow cytometric analysis of GC-
30 2spd(ts) cell line grown at 37°C for 16 generations
(Figure 3A), 24 generations (Figure 3B), 30 generations

(Figure 3C), compared with control mouse testis (Figure 3D).

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides immortalized and
5 conditionally immortalized non-tumorigenic germ cell
lines. Prior to the present invention, those skilled in
the art have for many years unsuccessfully attempted to
produce a germ cell line capable of being cultured _in vitro_ for an indefinite period of time. Such
10 immortalized and conditionally immortalized germ cell
lines are useful as an _in vitro_ model to study
spermatogenesis. They are also useful as a model to
identify and manipulate the factors that control germ
cell proliferation and differentiation. In addition, _in
15 vitro_ germ cell lines are induced to differentiate into
mature sperm cells, which in turn are used for _in vitro_
fertilization to produce transgenic animals, such as
transgenic mice.

As used herein the term "immortalized cell
20 line" refers to a cell line in which a foreign gene
encoding for an immortalizing molecule is integrated into
a naturally occurring cell line such that the line can
then be cultured _in vitro_ for an indefinite period of
time. As used herein, "immortalized or permanent cell
25 line" refers to cells that can be cultured _in vitro_ for
at least one month. Such immortalized or permanent cell
lines are viable whether continuously cultured _in vitro_
or stored according to methods known in the art. As used
herein the term "immortalized germ cell line" refers to a
30 germ cell line which has been immortalized but which is
non-tumorigenic and non-malignant.

As used herein the term "conditionally
immortalized cell line" refers to an immortalized cell

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line in which the immortalizing molecule can be activated
or inactivated under appropriate conditions. When the
immortalizing molecule is activated, the cell line
proceeds with mitotic proliferation, and when the
5   immortalizing molecule in inactivated, the cell line
proceeds to differentiate. A conditionally immortalized
cell line can be constructed in a number of ways. A
foreign gene encoding a temperature-sensitive
immortalizing molecule, such as the polyoma large T
10   antigen, can be integrated in the genome of a cell. The
resulting cell line can be grown at temperatures at which
the immortalizing molecule is active, or at which the
molecule becomes inactive, such as between about 38°C and
40°C. In another embodiment, an immortalizing molecule
15   can be operably linked to an inducible promoter that
drives the expression of the immortalizing molecule.
Alteratively, a cell line can be cotransformed with a
gene encoding an immortalizing molecule and a gene
encoding a molecule which binds with and inactivates the
20   immortalizing molecule under certain conditions. For
example, a cell line can be transformed with an
immortalizing large T antigen, and a temperature-
sensitive antiproliferative protein such as p53 capable
of inactivating the large T antigen in the cell. As used
25   herein, the term "conditionally immortalized germ cell
line" refers to a germ cell line which has been
conditionally immortalized but which is non-tumorigenic
and non-malignant.

30   As used herein the term "antiproliferative
protein" refers to tumor suppressor gene products such as
cellular phosphoprotein p53 or Rb which have been found
to complex with the transforming proteins of DNA tumor
viruses in transformed cells. The p53 protein has been
35   identified as associated with the large T antigen in SV-
40 transformed cells (Lane et al. Nature (London) 278:
261-263 (1979), and Linzer et al., Cell 17:43-52 (1979)),

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the E1b 55 kDa protein of adenovirus (<u>Sarnow et al. Cell</u> <u>28</u>:387-394 (1982)), and the E6 protein of papillomavirus (Werness et al. <u>Science 248</u>: 76-79 (1990)). Exogenous wild type (wt) p53 has been shown to have

5   antitransforming activity (Finlay et al. <u>Mol Cell Biol 8</u>: 531-539 (1988)) when co-transfected with transforming proteins. In addition, wtp53 was found in mutated form, in reduced amounts, or to be absent in a wide variety of naturally occuring human tumors.

10   As used herein, the term "crisis" refers to a primary cell culture which is undergoing a number of cell divisions during which most of the secondary cells die and disintegrate; the remaining cells have the potential to give rise to an established cell line. As used herein

15   the term "precrisis" refers to the state of a primary cell culture before crisis.

             According to the present invention, a germ cell line is immortalized by integrating a plasmid containing a gene encoding for an immortalizing molecule into the

20   chromosome of a naturally occurring germ cell. For example, a germ cell from the testis of a male animal can be transfected with the plasmid, pSV3-neo (ATCC No. 37150), which contains a gene encoding for the SV40 large T antigen. The SV40 large T antigen is known to be an

25   immortalizing molecule. Other plasmids containing genes encoding for large T antigens or other immortalizing molecules having immortalizing properties similar to the large T antigen, such as E,A (adenovirus), c-myc, c-jun, h-ras and v-scr, which are all well known in the art, can

30   also be used. The SV40 large T antigen, a nuclear factor that normally regulates SV40 viral replication, is used to immortalize the germ cells at a particular stage of differentiation by virtue of its replicative functions. More particularly, the large T antigen locks the cells at

35   the developmental and gene expression stage of early

meiosis, while forcing the cells to cycle once again by
mitosis. Other large T antigens useful in the present
invention include, for example, polyoma virus large T
antigens and temperature-sensitive polyoma large T

5   antigens, as described in Linder et al., Exp. Cell Res.
191:1-7 (1990) and J. Chou, PNAS USA 75:1403-1413 (1978).

The methods for cultivating germ cell lines for
the purpose of immortalizing them differ from previous
attempts at the long term culture of non-tumorigenic germ

10 cells is that the germ cells were grown in the presence
of a monolayer of the somatic testicular cells prior to
immortalizing with the large T antigen. Thus, the germ
cells were cultured for a few days in a seminiferous
tubule-like environment prior to immortalization.

15   Germ cells can be immortalized at defined
stages of differentiation including, for example,
primitive type A, type A or B spermatogonia, primary
spermatocytes or secondary spermatocytes. Germ cells can
be pre-selected at a desired differentiation stage before

20 an immortalizing plasmid is incorporated into the genome.
For example, germ cells taken from a testis corresponding
to the different stages of differentiation can be
separated using unit gravity sedimentation procedures,
which are well known in the art. For example, the STA-

25 PUT™ sedimentation procedure (Johns Scientific, Toronto,
Canada) can be used, which is described in Romrell et
al., Dev. Biol. 49:119-131 (1976). The type of
immortalized germ cell line to be obtained therefore
depends on the amount of differentiation a germ cell has

30 undergone at the time the cell is extracted from a male
animal.

An immortalized or conditionally immortalized
germ cell line can be characterized according to its
stage of differentiation by detailed microscopic and

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electron microscopic examination. The presence or
absence of stage-specific protein markers can also be
used. For example, a germ cell line can be characterized
as a primary spermatocyte by its expression of testis-
5   specific lactate dehydrogenase (LDH-C) and the testicular
isoform of cytochrome c (cytochrome c_t). The immortalized
germ cell line designated as GC-1 is believed to be in a
transition stage between spermatogonia and the primary
spermatocyte stage. Studies indicate that the GC-1
10   expresses cytochrome c_t and LDH-C. The conditionally
immortalized germ cell lines designated GC-2spd(ts) and
GC-3spd(ts) can be characterized as in the secondary
spermatocyte stage. At the permissive temperature of
37°C, LDH-C production was enhanced, although cytochrome
15   c_t production is reduced. Thus, a further aspect of the
present invention is a method of producing such
isoproteins in vitro by culturing immortalized or
conditionally immortalized germ cells expressing such
isoproteins, and harvesting the isoproteins by methods
20   known in the art such as described, for example, in
Goldberg, J. Biol. Chem. 247:2044-2048 (1972).

In another aspect of the present invention,
conditionally immortalized germ cell lines are
constructed in which the immortalizing molecule can be
25   modulated under certain conditions to allow the cell
lines to either proliferate or undergo differentiation in
vitro. When the immortalizing molecule is activated, the
cell line proceeds with mitosis; when the immortalizing
molecule is inactivated, the cell line proceeds with
30   differentiation. Constructing conditionally
immortalized germ cell lines can be accomplished for
example, by transfecting the primary germ cell with a
gene encoding a temperature-sensitive immortalizing
molecule. When the conditionally immortalized cell line
35   is kept at permissive temperatures, the expressed
immortalizing molecule is active, and the cell lines

proliferate. At a non-permissive temperature, the
immortalizing molecule is inactivated, and the cells can
proceed through differentiation. In another embodiment,
an immortalizing molecule is operably linked to an
5   inducible promoter that drives the expression of the
immortalizing molecule.

        Alternatively, activation and deactivation is
controlled by a gene encoding a molecule which binds to
and inactivates the immortalizing molecule inside the
10   cell under certain conditions. Such cell lines are
constructed by cotransfecting both a gene encoding for an
immortalizing molecule and a gene encoding for an
antiproliferative molecule capable of binding the
immortalizing molecule under certain conditions. For
15   example, primary germ cell populations enriched in
secondary spermatocytes were cotransfected with a gene
encoding an immortalizing molecule and a gene encoding an
antiproliferative molecules capable of binding the
immortalizing molecule. In one preferred embodiment,
20   primary mouse germ cells were cotransfected with the SV40
Large T (LTAg) antigen gene and the gene encoding for a
temperature-sensitive (ts) mutant of p53, to obtain cell
lines in which both moelcules were expresssed. Two
secondary spermatocyte cell lines, GC-2spd(ts) and GC-
25   3spd(ts) were established in which an excess of p53 was
able to bind LTAg at the permissive temperature, thus
reducing the proliferative effects of LTAg. One of these
cell lines in particular, GC-2spd(ts), was shown to be
capable of differentiating and undergoing meoisis in
30   vitro. Differentiation was characterized by
immunocytochemical and morphological observation, as
described in detail in Example V.

        In another aspect, the present invention
relates to methods of controlling the proliferation or
35   differentiation of a conditionally immortalized germ cell

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line by activating or deactivating the expressed
immortalizing molecule. Proliferation of an immortalized
germ cell can be facilitated by the presence of the
immoralizing molecule, while differentiation can be
5   facilitated by the inactivation of the molecule. Methods
include the use of a temperature-sensitive immortalizing
molecule, such as temperature sensitive large T antigens,
which are well-known in the art. Methods also include
the linkage of a gene encoding an immortalizing molecule
10   to an inducible promoter which drives the expression of
the immortalizing molecule, or the cotransfection of a
primary cell with a gene encoding the immortalizing
molecule and with a gene encoding a molecule capable of
inactivating the immortalizing molecule under certain
15   conditions.

The present invention also relates to
immortalized somatic cell lines derived from non-
tumorigenic testicular cells of an animal, particularly
Sertoli, peritubular and Leydig cell lines, which are
20   capable of being cultured in vitro for at least one month
and preferably indefinitely.

A few somatic cell lines with characteristics
of Sertoli, peritubular and Leydig cells have been
previously cultured in vitro. For example, cultured
25   Leydig-like cells are described in Mather, Biol. Reprod.
23:243-252 (1980) and are available under the ATCC Nos.
CRL 1714 and CRL 1715, while Yasumura et al., Science
154:1186 (1966) describes Leydig-like tumor cell lines
(ATCC No. CCL 83). Cultured peritubular-like cell lines
30   are described in Mather et al., J. Ultrastructural Res.
87:263-274 (1984). However, these known cell lines
differ from the somatic cell lines of the present
invention in several ways. The known somatic cell lines
are mostly derived from spontaneous tumors or other
35   abnormal tissues, whereas the cell lines of the present

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invention display normal morphology and are considered
non-tumorigenic.

The present cell lines have been immortalized
or conditionally immortalized from cells of the same
5   developmental stage using a consistent and minimal
genetic change, that is, introduction and stable
integration of the pSV3-neo plasmid, and expression of
the SV40 large T antigen gene, and for some cell lines
the p53 gene as well. Previous attempts at long term
10 cultures, on the other hand, have involved combining at
least two cell types of the somatic cell environment or
co-cultures of Sertoli and germ cells as the primary
cultures.

Furthermore, the viability of these known
15 primary co-cultures seldom exceeds fifteen days, whereas
the immortalized somatic cell lines of the present
invention have been cultured for more than 2 years. The
known long-term cultures of such somatic cells have been
derived generally from tumor cells compared with the
20 immortalized non-tumorigenic cell lines of the present
invention.

When cells from each of the immortalized
somatic and germ cell lines are plated together, they
25 reaggregate to form in vitro seminiferous tubule-like
structures. The germ cells appear in the center of these
formations, while Sertoli, peritubular and Leydig cells
establish borders between these germ cell cores, similar
to the structures produced by non-tumorigenic, freshly
30 isolated testicular cells. Thus, immortalized germ cells
can be cultured in the presence of fresh or immortalized
somatic cells within the in vitro seminiferous tubule-
like environment. Conversely, immortalized somatic cells
can be used to grow and induce the differentiation of
35 non-immortalized germ cells.

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The formation of the tubule-like structures can be visually monitored under microscopic observation as well as aided by detectable markers, such as fluorescent dyes. Cells from at least one immortalized cell line can

5 be labeled with a fluorescent dye, such as DiI (D-282) or DiO (D-275) (Molecular Probes, Eugene, OR), or other known labels or methods as described, for example, in Honig et al., J. Cell Biol. 103:171-187 (1980). Other dyes that can be used include 1% trypan blue, trypan red

10 or india ink in H₂O, 0.025-0.25% methylene blue, Tanus green B or neutral red.

The immortalized and conditionally immortalized cell lines of the present invention can be used for a variety of purposes. For example, paracrine and

15 autocrine factors responsible for germ cell differentiation, nutrient requirements, steroidogenic pathways and conditions that will allow non-tumorigenic germ cells to be cultured in vitro can be studied using the tubule-like structures as an in vitro model. In

20 addition, immortalized germ cells that have differentiated to the spermatid stage or beyond, thus completing the second meiotic division, can be used to fertilize oocytes in vitro by nuclear transfer or cell fusion techniques. Such techniques are well known in the

25 art such as described, for example, in Palmiter et al., Ann. Rev. Genet. 20:465-99 (1986). If differentiation continues to the mature sperm stage, the conventional in vitro fertilization procedures can be used. Such procedures can be used to produce transgenic mice, for

30 example. Additionally, germ cells from a prized animal can be immortalized at a particular stage, frozen or cultured for an indefinite period and later used for in vitro fertilization. Thus, the present invention can be used for breeding certain desirable characteristics.

15

The following examples are intended to
illustrate but not limit the following invention.

EXAMPLE I

Immortalization of Cells From Immature Mouse Testis

5   Testes from four 10-day old Balb/C mice were
collected aseptically in serum-free CMRL-1066 culture
medium (Gibco, Bethesda, MD), rinsed in 0.1 M phosphate
buffered saline (PBS) and treated with collagenase (1
mg/ml in PBS) (Boehinger-Mannheim) for 15 minutes at room
10   temperature. The medium was replaced by PBS containing
DNase (10 ug/ml)(Sigma Chemical Co., St. Louis, MO) and
the mixture pipetted up and down several times until a
cell suspension was obtained. Cells were washed in PBS,
then layered onto a discontinuous Percoll (Pharmacia,
15   Piscataway, NJ) gradient composed of 4 layers with
densities of 1.055, 1.045, 1.035 and 1.025, respectively,
as described in Schumacher et al., FEBS LETT. 91:333-38
(1978). Centrifugation was carried out at 200 x g for 20
minutes in a bench-top centrifuge. Cells were
20   distributed into 3 bands, corresponding to the 3
interphases of the Percoll gradient. Based on their
morphology, adherence potential and growth
characteristics, band A (density = 1.030) was enriched in
spermatogonia and primary spermatocytes, band B (density
25   = 1.040) in Sertoli and peritubular cells, and band C
(density = 1.050) was enriched in Leydig and endothelial
cells, both obtained from the same testes. Cells of each
band were cultivated separately in tissue culture flasks
(Falcon). Cell survival was optimal (10 days for
30   spermatogonia, more than 20 days for somatic cells) in
CMRL-1066 medium enriched with 80 μg/ml insulin, 3 μg/ml
transferrin, 80 μg/ml ascorbic acid and 13% inactivated
fetal calf serum.

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Cells of bands B and C were cultivated for 2
weeks until monolayers were formed. These bands, that
contain all four cell types although in different
relative proportions, reconstituted tubuli-like
5   structures in vitro, with germ cells always homing to the
center of these formations. These monolayers (in 25 cm²
flasks) were transfected by the calcium phosphate
procedure as described in Gorman et al., Mol. Cell. Biol.
2:1044-1051 (1982), incorporated herein by reference,
10   with 25 μg of the pSV3-neo plasmid (ATCC #37150) that
contains the genes coding for the immortalizing SV40
large-T antigen and resistance to neomycin (selection
marker). After transfection, cells were given fresh
culture medium. Four days later, G418 (geneticin, Gibco)
15   was added at a concentration of 200 μg/ml of active
substance. At weekly intervals, the selection medium was
changed until colonies appeared.

Cell colonies surviving the selection procedure
were picked by gentle scrapping and cultivated separately
20   in culture medium. Immortalized colonies were obtained
at a frequency of about 10^{-6}. No colonies survived when
parallel cultures were transfected with the non-
immortalizing plasmid control, pSV2-neo, (ATCC # 37198)
which lacks the SV40 large T antigen. Since the
25   resulting immortalized colonies were not pure, extensive
clonings by limiting dilution (4 to 6 single-cell
clonings per cell line) were carried out, yielding a
total of 49 lines. These cell lines have now been
continuously cultured for several generations over a
30   period of 2.5 years and are considered permanent cell
lines.

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EXAMPLE II
Integration of the SV40 Large T Antigen Gene

      After trypsinization, cells were washed once in
PBS and pelleted by centrifugation. Genomic DNA was
 5 recovered from cell pellets by treatment with 1% SDS and
50 µg/ml proteinase K in TE buffer (0.01 Tris, 0.001 M
EDTA, pH 7.5). DNA was extracted with phenol and
chloroform and recovered by ethanol precipitation
according to well known standard methods as described in
10 Sambrook et al., Molecular Cloning: A laboratory manual,
Cold Spring Harbor Laboratory Press (N.Y. 1989),
incorporated herein by reference. Fifteen µg of genomic
DNA and 1 ng of pSV3-neo DNA (positive control) were
digested with Bam HI in order to excise the large T
15 antigen gene. The samples containing PG3 peritubular
cell DNA, SH2 Sertoli cell DNA, LAB2 Leydig cell DNA and
germ cell DNA were electrophoresed in 0.8% agarose gel
and transferred onto a nitrocellulose filter. The filter
was hybridized with a ^{32}P-labelled Bam HI fragment of the
20 pSV3-neo.

      All the permanent cell lines integrated the
SV40 large T antigen gene in their genome as shown by
conventional Southern blot analysis of the cellular high
molecular weight DNA. A 3.3 kb fragment was detected
25 that corresponds to the large T antigen gene. These
results confirm that the immortalizing gene has been
integrated in the genome of these cells. In addition,
these cells show expression of the large T antigen with
the typical nuclear localization, as shown by
30 immunocytochemistry.

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<u>EXAMPLE III</u>
<u>Characterization of the SV40 Large T</u>
<u>Antigen Immortalized Cell Lines</u>

The forty-nine permanent cell lines obtained
5 were characterized further according to their morphology,
presence of histochemical and immunohistochemical
markers, their lack of growth in soft agar and their
ability to reconstitute tubule-like structures when the
cells were combined <u>in vitro</u>. Table 1 summarizes the
10 results obtained in these characterization studies.

TABLE 1

	SERTOLI	LEYDIG	PERITUBULAR	GERM CELLS
15 # of clones	8	22	16	3
growth in soft agar	1 of 8	3 of 22	5 of 16	0
morphology of non-transformed clones	normal	normal	normal	normal
20 immunohistochemical markers	ND	3 ß-ol DH; Alkaline phos.	Desmin; Alkaline phos.	Cytochrome C_t;
Morphogenic potential for 25 tubule-like structures	conserved	conserved	conserved	conserved

The 49 permanent cell lines have been designated
as follows: <u>S</u>ertoli (SC5, SH2, SC1, SF7, SG4, SB7, SC72
30 and SE121), <u>p</u>eritubular (PE8, PG3, PE11, PG52, PB51, PF71,
PG121, PH121, PD32, PE102, PB83, PD103, PC111, PD122, PA122
and PH12), <u>L</u>eydig (LJG1, LEC2, LAC11, LCF6, LB32/G4,
LB32/E3, LIF8, LB32/B7, LFG6, LFA2, LBC12, LEA7, LEB10,
LED2, LFA6, LBB7, LAB2, LHD7, LGC12, LB1ro, LAH7 and LFB3)
35 and <u>G</u>erm <u>c</u>ell (GC-1, GC-2 AND GC-3).

A.   Test for Malignant Transformation

        The use of oncogenes to immortalize cells is
known to result occasionally in the malignant
transformation of the cells as evidenced by their ability
5   to grow in soft agar and/or in nude mice. To determine
whether the immortalized cells had undergone malignant
transformation, cell monolayers were first trypsinized,
treated with 1 mg/ml collagenase in PBS until a single-
cell suspension was obtained and washed several times in
10   PBS. Soft agar cultures were performed following the
method of Hamburger and Salmon, Science 197:461 (1977),
incorporated herein by reference. Briefly, $1x10^6$ cells
were resuspended in warm CMRL-1066 medium fortified as
described in Example I and containing 0.3% agar noble
15   (Difco Laboratories, Detroit, MN). The mixture was
poured onto an underlayer previously prepared in 60 mm
diameter culture dishes. The underlayer was made of (.6%
agar in McCoy's medium 5a completed with 1% sodium
pyruvate, 42 μg/ml L-serine, 1% glutamine (200 mM), 0.15%
20   tryptic soy broth, 1% penicillin/strep and 13%
inactivated fetal calf serum. Cultures were incubated at
37°C and 5% CO_2 for at least one week before assessment of
clonal proliferation. Only between 12.5% and 32.5% of
the immortalized clones were able to grow in soft agar,
25   i.e., 1 out of 8 Sertoli (SC72), 3 out of 22 Leydig
(LFA2, LED2 and LAH7) and 5 out of 16 peritubular (PG52,
PB51, PF71, PE102 and PB83) cells, while the majority of
the immortalized somatic cell lines and our immortalized
germ cell clone (GC-1) had retained a normal growth
30   behavior including contact inhibition. Contact
inhibition is a property of non-tumorigenic, non-
malignant cells in which the cells proliferate until they
come into contact with adjacent cells to establish a
monolayer. Malignant cells, on the other hand, will not

20

arrest at this stage and will continue to grow on top of
each other.

B.   Electronmicroscopic Analysis

        The cells were also analyzed by
5   electronmicroscopy. The cell lines were fixed in 2.5%
glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, and
postfixed in 1% OsO_4 followed by saturated
thiocarbohydrazide and a second OsO_4 treatment. The fixed
cells were stained with 2% uranyl acetate and embedded in
10   an Epon 812 mixture (Shell Epon Resin 812, E.V. Roberts &
Associates, Palo Alto, CA). Sections were obtained with
a Reichert Ultracut E ultramicrotome and viewed with an
Hitachi H-600 electron microscope.

        Electronmicrographs show distinctive features
15   of Sertoli and peritubular cells. Characteristic
features of Sertoli cell line SH2 include lipid and
glycogen inclusions and an abundant agranular reticulum
as well as numerous spherical and elongated mitochondria
exhibiting transverse cristae of orthodox lamellar
20   configuration. Characteristic features of the
peritubular cell line PG3 include longitudinal
microtubules, abundant microfilaments and secretion and
deposition of a collagen-rich extracellular matrix.

        Electronmicrographs show incomplete cytokinesis
25   in cultures of the immortalized germ cell line GC-1. At
any one time, approximately 5% of the GC-1 cells can be
found in this state. This incomplete cytokinesis is not
observed with the somatic cell lines, and resembles the
in vivo situation where primary spermatocytes undergo
30   meiosis connected by cytoplasmic bridges in a syncytium-
like fashion.

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C.   Immunohistochemical Characterization

          To characterize the specific markers expressed
by the different immortalized cell types, the
immortalized cells were cultured to confluency in Lab-Tek
5   chamber slides (Nunc, Naperville, IL) with complete D-MEM
medium and fixed with cold methanol. Antigens were
revealed by using an immunoperoxidase technique according
to the manufacturer's directions (Vectastain ABC kit,
Vector, Burlingame, CA). The primary antibodies used
10   were mouse monoclonal anti-SV40 large T antigen (1:100 in
PBS) (Oncogene Science, Manhasset, NY), and mouse
monoclonal anti-desmin (1:100 in PBS) (Amersham,
Arlington Heights, IL). The rabbit polyclonal and mouse
monoclonal antibodies against the testis-specific LDH-C
15   isozyme and the rabbit polyclonal antibodies against the
testis-specific isoform of cytochrome c (designated
cytochrome c_t) were supplied by Dr. Erwin Goldberg
(Northwestern University, Illinois). Counterstaining was
performed with hematoxylin, fast green or neutral red.

20   Two histochemical markers were detected. The
3β-hydroxysteroid dehydrogenase (3β-HDH) assay, an enzyme
that converts pregnenolone to progesterone and is
characteristic of Leydig cells, was performed according
to the method described by Wiebe et al. in Endocrinology
25   98:505-513 (1976), using dehydroepiandrosterone, β-NAD
and nitro blue tetrazolium. After the reaction was
completed, cells were fixed in 4% formalin in PBS, pH 7.2
(buffered formalin) and counterstained with 0.1% neutral
red. For detection of total alkaline phosphatase
30   activity, Leydig and peritubular cells were first fixed
with buffered formalin, stained with Naphthol As-MX
phosphate (Sigma Chemical Company, St. Louis MO) and Fast
Violet B salt (Sigma Chemical Co.) by a well known
procedure described in Wiebe, Endocrinology 98:505-513
35   (1976), which is incorporated herein by reference, and

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counterstained with hematoxylin according to standard
conventional procedures.

     Both peritubular and Leydig cells were found
positive for alkaline phosphatase. The alkaline
5   phosphatase staining in peritubular cells was
preferentially associated with spindle-like growth of the
cells and was much weaker when the cells were more sparse
and appeared with round morphology. The color of the
alkaline phosphatase reaction product in peritubular and
10 Leydig cells was consistently different, suggesting that
different isozymes are expressed. Preliminary Northern
blot results indicate that this may be the case. These
results were confirmed by reverse-transcriptase
polymerase chain reaction amplification (RT-PCR) of poly
15 A + mRNA extracted from these cell types, using isozyme-
specific oligonucleotide primers, as previously
described.

     Sertoli cells were negative histochemically for
alkaline phosphatase as was the germ cell line GC-1.
20 Peritubular cells were selectively stained with a
monoclonal antibody against desmin. The murine anti-
desmin antibody was obtained from Amersham (Arlington
Heights, Illinois) and is described in Anthony et al.,
Biol. Reprod. 40:811-823 (1989). Similarly, Leydig cells
25 exhibited the characteristic intracytoplasmic dotted
staining for the enzyme 3ß-HDH. These cell-type
characteristics were preserved when all four immortalized
cell lines were plated together and reconstituted tubule-
like structures as described in Example IV.

30   The germ cell line, GC-1, was negative for the
markers previously identified in the somatic cell lines.
It, however, has been shown to express the testicular
isoform of cytochrome c. Initial studies indicate that
these cells can also express the testis-specific lactate

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dehydrogenase (LDH-C). These isozymes are recognized as being specific for the spermatogenic lineage and expressed from the preleptotene stage of germ cell differentiation onward. The GC-1 line is a permanent

5   cell line capable of expressing isoproteins in culture. No other permanent cell line is known to express these isoproteins in vitro. The immunohistochemical staining of the GC-1 cell line using a monoclonal antibody against LDH-C and a monospecific rabbit polyclonal antibody to

10   the testicular isoform of cytochrome c demonstrated the reactivity of the antibody to LDH-C and cytochrome c. The antibodies against LDH-C and cytochrome c_t are described and identified in Goldberg et al., Science 196:1010-1012 (1977), incorporated herein by reference.

15   The designation of primary spermatocyte usually implies that the cell in question has stopped dividing by mitosis, has differentiated and is entering the 1st meiotic division. Both the LDH-C and cytochrome c_t gene products are known to start being expressed at the

20   preleptotene/leptotene transition. By immortalizing the germ cell line at this stage, the large T antigen expression (a nuclear factor that normally regulates SV40 viral replication) has "locked" the cells at the developmental, and gene expression, stage of early

25   meiosis while forcing the cells to cycle again by mitosis resulting in the proliferation of the cells and continued expression of the isoproteins.

                              EXAMPLE IV
              Formation of In Vitro Tubule-like Structures

30   One characteristic of the in vitro immortalized cell lines of the present invention is their ability to associate and reconstitute seminiferous tubule-like structures when plated together. The primary spermatocyte line GC-1 appears in the center of these

formations while Sertoli, peritubular and Leydig cells
contribute to establishing the borders between the germ
cell cores.

         At least one of the somatic cell components is
5   labeled with a fluorescent marker to facilitate following
    the distribution of the individual somatic cell
    components. A lipophilic carbocyanine membrane probe,
    DiI (D-282), was used to label the somatic cell lines.
    The fluorescent dye, DiO (D-275), can also be used to
10 label the cell lines. The cells were incubated for 1
    hour at 37°C in serum-free D-MEM containing 10 µg/ml DiI.
    The cells were subsequently washed twice in PBS prior to
    the co-culture experiments. The _in vitro_ structures
    produced by non-tumorigenic, freshly isolated testicular
15 cells were similar to those produced by the immortalized
    cell lines.

                        EXAMPLE V
                 Co-Transfected Cell Lines

         Primary mouse germ cells, obtained as described
20 below, were cotransfected plasmid pSV3-neo (ATCC NO.
    37150), containing the LTAg gene and the neo^r gene, and
    the plasmid LTRp53cG9, containing the gene encoding for
    temperature sensitive [val^{135}]p53 (described in Fukasawa
    et al. _Mol and Cell Biol 11_: 3472 -3483 (1991)),
25 incorporated herein by reference. The (val^{135})p53 is non-
    functional at 39°C, and functional at 37°C and 32°C. As
    described in detail below, at the non-permissive
    temperature of 39°C, the co-transfected cell lines
    continued to undergo mitosis because the newly introduced
30 p53 gene was inactive and LTAg retained its immortalizing
    ability by binding to and inactivating the endogenous
    wtp53. At the permissive temperatures of 37°C and 32°C,
    excess p53 bound to LTAg and reduce or abolish its
    immortalizing properties.

25

A.   <u>Obtaining Cell Lines</u>

        A single cell suspension was obtained from
decapsulated testes of sexually mature Balb/c mice as
previously described above in Example I above. A cell
 5   fraction enriched with spermatocytes was then isolated
using the STA-PUT™ (Johns Scientific, Toronto, Canada)
gravity sedimentation procedure at 4°C according to the
procedure described in Romrell et al, <u>Dev. Biol. 49</u>: 119-
131 (1976), and Peden et al, <u>Virol 168</u>, 13 (1989), each
10   incorporated herein by reference. Spermatocytes were
cultivated in 60 mm tissue culture dishes (Falcon) and
completed CMRL-1066 medium until a monolayer was formed.

        Cotransfection was performed with two plasmids,
the pSV3-neo plasmid which contains the LTAg gene and the
15   neo^r gene, and the LTRp53cG9 plasmid which contains the
temperature sensitive [val^{135}]p53gene, and which was
previously described in Michalowitz et al. <u>Cell 62</u>, 671-
680 (1990) and Martinez et al. <u>Genes & Develop. 5</u>, 151
(1990), each incorporated herein by reference, and was
20   provided by Dr. Channing Der, University of North
Carolina, Chapel Hill, North Carolina. Cotransfection of
12.5μg pSV3-neo (ATCC #37150) and 12.5μg LTRp53cG9 per
dish was performed by the calcium phosphate method
described in Sambrook et al., <u>Molecular Cloning: A</u>
25   <u>Laboratory Manual</u>, Cold Spring Harbor Laboratory Press,
Cold Spring Harbor, N.Y. (1989), incorporated herein by
reference. G418 (Geneticin, Gibco, Chagrin Falls, OH),
at a concentration of 200 μg/ml of active substance, was
used for the selection of neomycin resistant colonies.

30   After selection with G418, two cell clones,
designated GC-2spd(ts) and GC-3spd(ts) were obtained and
cultivated after crisis for a period of six months.

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B.   Characterizing the Cell Lines

       The two cells lines GC-2spd(ts) and GC-3spd(ts)
showed adherence in tissue culture and were contact
inhibited. No clonal proliferation was observed on soft
5   agar cultures, indicating that these cells are
immortalized but not transformed.

       The cells lines were characterized according to
morphology, growth characteristics, and
immunocytochemistry at various temperatures. To
10   determine the presence and location of LDH-C and
cytochrome c, immunocytochemical analysis was performed
as follows. The cells were grown at 37°C and 32°C to
monolayers on LABTEK slide chambers (Nunc, Inc.,
Naperville, IL) in D-MEM completed medium (as described
15   in Hofmann et al., Exp Cell Res 201, 417 to 435 (1992)),
fixed with cold methanol, washed in PBS and incubated in
PBS at 4°C for at least 2 days. Monolayers were stained
using a Vectastain ABC kit (Vector Laboratories,
Burlingame, CA) according to Example IIIC above using a
20   polyclonal antibody against the testis-specific LDH-C
isozyme, and rabbit polyclonal antibodies against the
testis specific isoform of cytochrome c (cytochrome c_t),
both supplied by Dr. Erwin Goldberg (Northwestern
University, Illinois), and counterstaining with
25   hematoxylin, fast green or neutral red. p53 was detected
with a monoclonal antibody against mouse wt53 (Ab-1,
clone 421, Oncogene Science, Manhasset, NY).

C.   Immunocytochemical Characteristics of Cell Lines at
Different Temperatures

30   39°C: As seen under phase contrast microscopy
at 39°C, the GC-2spd(ts) and GC-3spd(ts) cell lines were
large and spherical, and grew rapidly with a doubling
time of 18 hours. Specific markers for meiotic germ

cells, LDH-C and cytochrome c_t, were expressed at low
levels. p53 overexpression was confined to the
cytoplasm.

    <u>37°C</u>:   At 37°C, both cell lines grew more
5 slowly, with a doubling time of 24 hours. Figure 1 shows
staining patterns for p53, LDH-C, and cytochrome c_t for
GC-2spd(ts) and GC-3spd(ts) at 37°C (Figure 1A) and 32°C
(Figure 1B). At 37°C, LDH-C expression was enhanced, but
cytochrome c_t was not identifiable by immunocytochemistry,
10 as shown in Figure 1A. The p53 protein was expressed in
both the cytoplasm and the nucleus in both cell lines.

    At 37°C, groups of cells showed signs of
morphologic differentiation already visible at the light
microscopy level. In some cells of both lines, but
15 particularly in GC-2spd(ts), a dark granule appears at
one pole of the nucleus, whereas the cell cytoplasm at
the other pole of the nucleus become elongated.

    Morphological differentiation of GC-2spd(ts)
was examined by growing the cell lines to confluency in a
20 LabTek chamber and fixed with cold methanol. Periodic
acid-Schiff (PAS) staining was performed according to the
methods described in Sheenan et al. <u>Theory and Practice
of Histotechnology</u>. Battelle Press, Columbus, OH (1987).
Immunocytochemical staining was performed as described
25 above (Example III), using a monoclonal antibody, HS-63
(donated by Erv Goldberg, Northwestern University,
Evanston, IL), against the sperm acrosome antigen MSA-63
(as described in Liu et al, <u>Biol Repr 46</u>, 937 to 948
(1992). For electronmicroscopy, cell monolayers were
30 fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer,
pH 7.4, postfixed in OsO_4 and embedded in an epoxy resin
by usual methods. Ultrathin sections were stained with
uranylacetate and lead citrate, and visualized with a
JEOL 100 cx electron microscope.

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Electomicroscopic analysis confirmed the
presence of this structure, which is shown in the
electron micrograph of Figure 2C, and identified it as
the developing sperm acrosome. Figure 2 shows
5   morphological differentiation of the line GC-2spd(ts).
Figure 2A shows periodic acid-Schiff (PAS) staining of
the acrosomal granule in a round spermatid. This granule
is membrane-bound and appeared to be derived from the
Golgi apparatus. As expected, this structure shows
10 periodic acid-Schiff's staining (performed as described
in Hess, R.A. Biol. Repr 43, 525-542 (1992)), as is shown
in Figure 2A and is positive immunocytochemically with a
monoclonal antibody against a sperm acrosome antigen,
MSA-63, as is shown in Figure 2B. Figurd 2C shows an
15 electron micrograph of the acrosomel granule.

Flow cytometry analysis was performed on the
GC-2spd(ts) cell line grown at 37°C for several
generations as follows. Cells were cultivated to
confluency in 25 cm² tissue culture flasks (Falcon),
20 typsinized, fixed in cold methanol, and RNA degraded with
100 μg/ml RNase. Cell nuclei were then stained with 50
μg/ml propidium iodide and fluorescence recorded with a
EPICS Profile flow cytometer (Coulter). Cell cycle data
were analyzed with Multicycle software (Phoenix Flow
25 Systems).

Flow cytometry analysis of the GC-2spd(ts) cell
line performed as described above showed that at 37°C the
cells first exhibited a DNA content which is diploid (G1)
and tetraploid (G2), corresponding to a diploid cell
30 cycle. Figure 3A shows the 16th generation of cell in
culture, showing a diploid cell cycle only. However,
prolonged cultivation of these cells at 37°C (over 20
generations) resulted in the appearance of a third and
haploid peak, with flattening of the G2 values (Figures
35 3B and 3C). Figure 3B shows the 24th generation of the

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same cells, which show a diploid cell cycle, as well as
an extra haploid peak. Figure 3C shows the 30th
generation of the same cells, which shows an increase of
the percentage of haploid cells. Figure 3D shows control
5   mouse testis cells.

        Haploidy was shown only at 37°C, indicating
that the cell cycle has to be maintained in order for the
cells to complete the second meiotic division. It
appeared that certain cells belonging to a pool of
10 continually dividing secondary spermatocytes were able to
proceed through the second meiotic division and to
differentiate into spermatids. This phenomenon was
attributed to the behavior of the p53 protein at 37°C in
this cell line. The borderline temperature of 37° seemed
15 to allow the expression of both mutated and wt p53.

        In contrast, the GC-3spd(ts) cell line did not
progress into meiosis. In this line, p53 was expressed
almost exclusively in the cytoplasm (mutant form) at
37°C, and only in the nucleus (wt form) at 32°C. Since
20 at this temperature the cell cycle is blocked, the cells
were probably prevented from undergoing the second
meiotic division.

<u>32°C</u>
        At 32°C, in both lines, cell growth was slowed
25 progressively and the cells died after an average of 10
generations. This was attributed to the expression of
the wild-type exogenous p53 gene that, in excess, blocked
the immortalizing action of LTAg and arrested the cell
cycle. In these cells, p53 was expressed almost
30 exclusively in the nucleus. LDH-C and cytochrome ct
expression were markedly enhanced as is seen in Figure 1.
The germ cell line bearing only the LTAg, GC-1, continued
to proliferate at 32°C and maintained the same
morphological features, whereas GC-2spd(ts) and GC-

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3spd(ts) cells become spindle-like, exhibited a long
cytoplasmic prolongation and a much smaller cell body.
The number of cells bearing the acrosomic granule
increase from 3% at 37°C to 10% at 32°C. Moreover, by
5 cultivating these cells at 32°C in serum-free medium, at
least 30% of the cells produced the acrosomic granule.
Cells that were switched very early from 37°C to 32°C
never underwent meiosis but continued to differentiate
for 10 generations, whereas GC-2spd(ts) cells that
10 underwent meiosis at 37°C and were switched to 32°C died
very quickly, leaving intact the pool of proliferating
cells.

Therefore, by using the immortalizing ability
of LTAg under the conditional modulation of tsp53, cell
15 line GC-2spd(ts) was obtained, capable of serving as a
stem cell pool of spermiogenic cells. These cells
underwent meoisis in vitro and produced round and
elongated spermatids. This cell line provides a unique
system to study meiosis and germ cell differentiation in
20 vitro.

Although the invention has been described with
reference to the presently preferred embodiments, it
should be understood that various modifications can be
made without departing from the spirit of the invention.
25 Accordingly, the invention is limited only by the
following claims.

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We claim:

     1.   An immortalized germ cell line.

     2.   The immortalized germ cell line of claim 1, wherein said germ cell line is of murine origin.

5   3.   The immortalized germ cell line of claim 1, wherein said germ cell line is a spermatogonia, primary spermatocyte, secondary spermatocyte or spermatid.

     4.   The immortalized germ cell line of claim 10 3, wherein said germ cell line expresses a testicular isoform of cytochrome c and LDH-C.

     5.   The immortalized germ cell line of claim 1, wherein said germ cell line is GC-1.

     6.   A method for producing an immortalized 15 non-tumorigenic germ cell line comprising:
     (a)   growing a non-tumorigenic germ cell in the presence of non-tumorigenic Sertoli, peritubular and Leydig cells; and
     (b)   immortalizing said germ cell with an 20 immortalizing molecule.

     7.   The method of claim 6, wherein said immortalizing molecule is a large T antigen.

     8.   The method of claim 7, wherein said large T antigen is an SV40 large T antigen.

25   9.   The method of claim 6, wherein said immortalizing molecule is transfected into the genome of said germ cell to immortalize said germ cell.

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10. A method of producing a testicular isoform
of cytochrome c _in vitro_, comprising:
     (a) culturing the immortalized germ cell line
of claim 4 _in vitro_; and
     (b) harvesting said cytochrome c.

11. A method of producing a testicular isoform
of LDH-C _in vitro_, comprising:
     (a) culturing the immortalized germ cell line
of claim 4 _in vitro_; and
     (b) harvesting said LDH-C.

12. An immortalized somatic cell line derived
from a non-tumorigenic mammalian testicular cell capable
of being cultured _in vitro_ for at least one month.

13. The immortalized cell line of claim 11,
wherein said somatic cell line is a Sertoli, a
peritubular or a Leydig cell line.

14. A method for culturing a non-tumorigenic
germ cell _in vitro_ comprising contacting said germ cell
with immortalized Sertoli, peritubular and Leydig cells.

15. A method for culturing an immortalized
germ cell _in vitro_ comprising contacting said
immortalized germ cell with non-tumorigenic Sertoli,
peritubular and Leydig cells.

16. The method of claim 14, wherein said
immortalized germ cell is from the germ cell line GC-1.

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17. A conditionally immortalized germ cell
line capable of undergoing meiosis and differentiation in
vitro.

5   18. The conditionally immortalized germ cell
line of claim 16 wherein the germ cell line is a murine
testicular germ cell line.

19. The conditionally immortalized testicular
germ cell line of claim 17 wherein the germ cell line is
secondary spermatocyte.

10   20. The conditionally immortalized germ cell
line of claim 17 wherein the germ cell line comprises a
first foreign gene encoding an immortalizing molecule and
a second foreign gene encoding a temperature sensitive
molecule which inactivates the immortalizing molecule at
15 a temperature of 37°C or less.

21. The conditionally immortalized germ cell
line of claim 19 wherein the first foreign gene encodes
the large T antigen.

22. The conditionally immortalized germ cell
20 line of claim 19 wherein the second foreign gene encodes
a molecule which binds to the immortalizing molecule is
an antiproliferative protein capable of binding to the
immortalizing molecule inside the cell.

23. The conditionally immortalized germ cell
25 line of claim 21 wherein the antiproliferative protein is
p53.

24. The conditionally immortalized germ cell
line of claim 22 wherein the cell line is GC-2spd(ts) or
GC-3spd(ts).

34

25. A method for preparing an immortalized germ cell line, comprising integrating a gene encoding an immortalizing molecule into a germ cell.

5 26. The method of claim 24, wherein the gene encodes for the large T antigen.

27. A method for preparing a conditionally immortalized germ cell line comprising co-integrating a gene encoding an immortalizing molecule and a gene encoding molecule which inactivates the immortalizing
10 molecule inside the transformed cell under certain conditions.

28. The method of claim 26 wherein the immortalizing molecule is a large T antigen.

29. The method of claim 26 wherein the
15 inactivating molecule is a temperature sensitive antiproliferation molecule.

30. The method of claim 28 wherein the antiproliferative molecule is temperature-sensitive p53.

31. A method of producing a testicular isoform
20 of lactate dehydrogenase in vitro, comprising:
    (a) culturing the germ cell line of claim 19 at a temperature of 37°C or less; and
    (b) harvesting said lactate dehydrogenase.

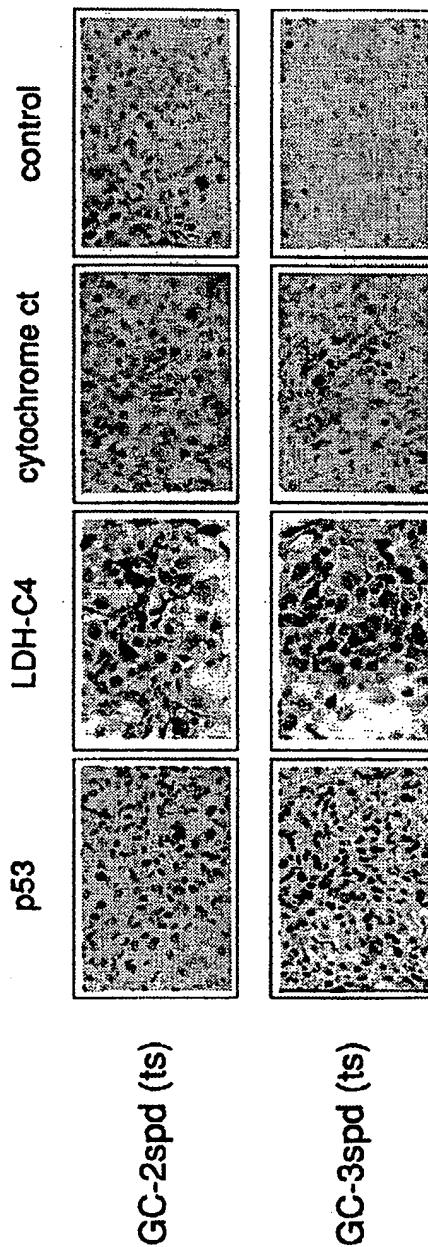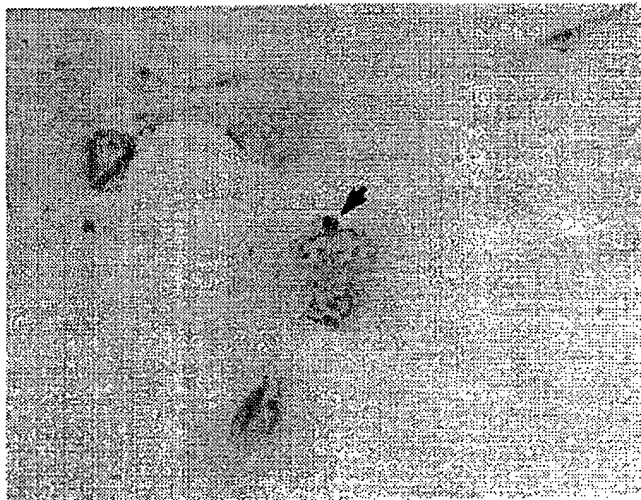**FIG. IA**

37°C

FIG. IB

32°C
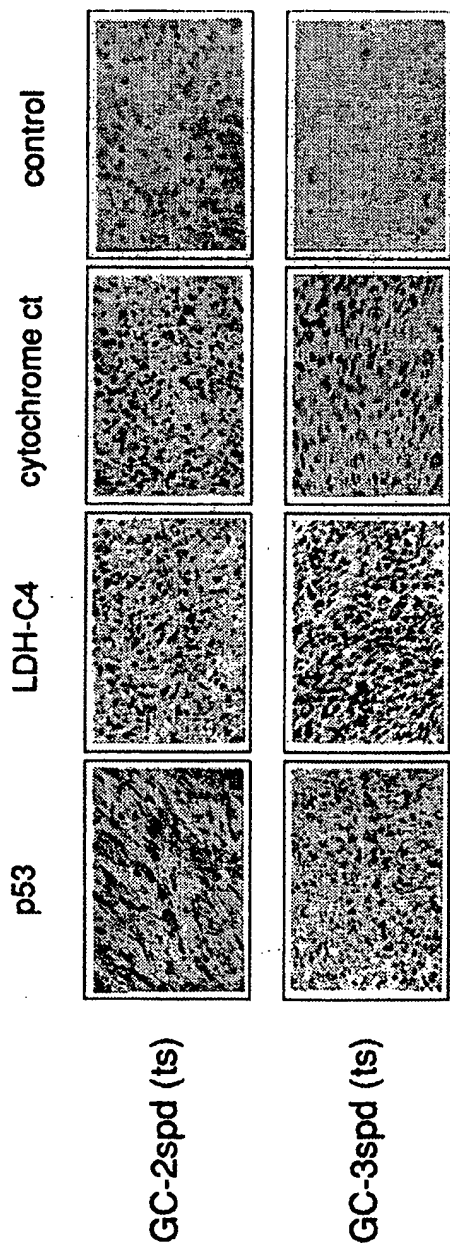
FIG. 2A
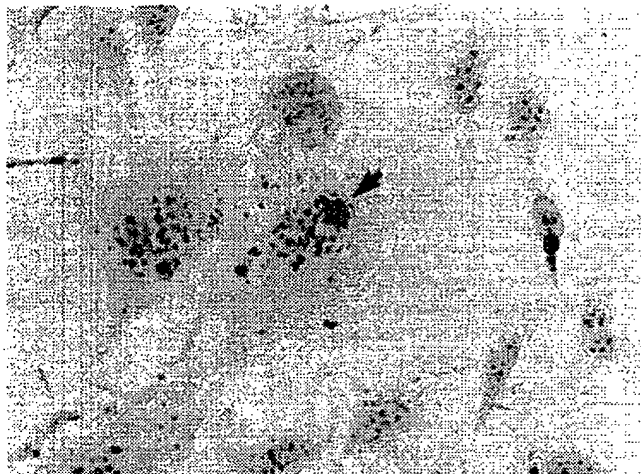
FIG. 2B
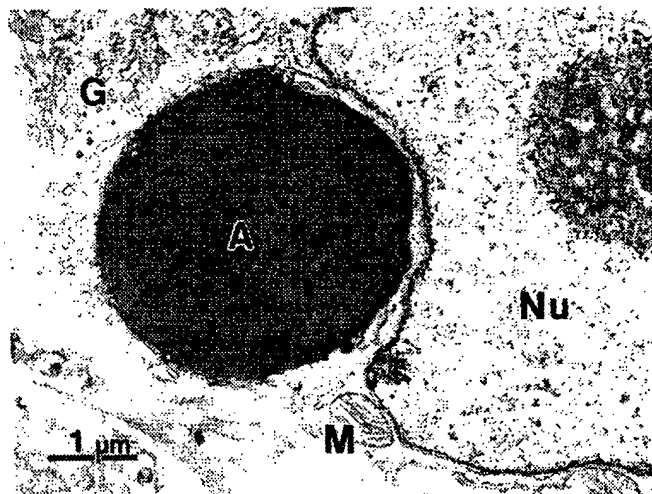
FIG. 2C
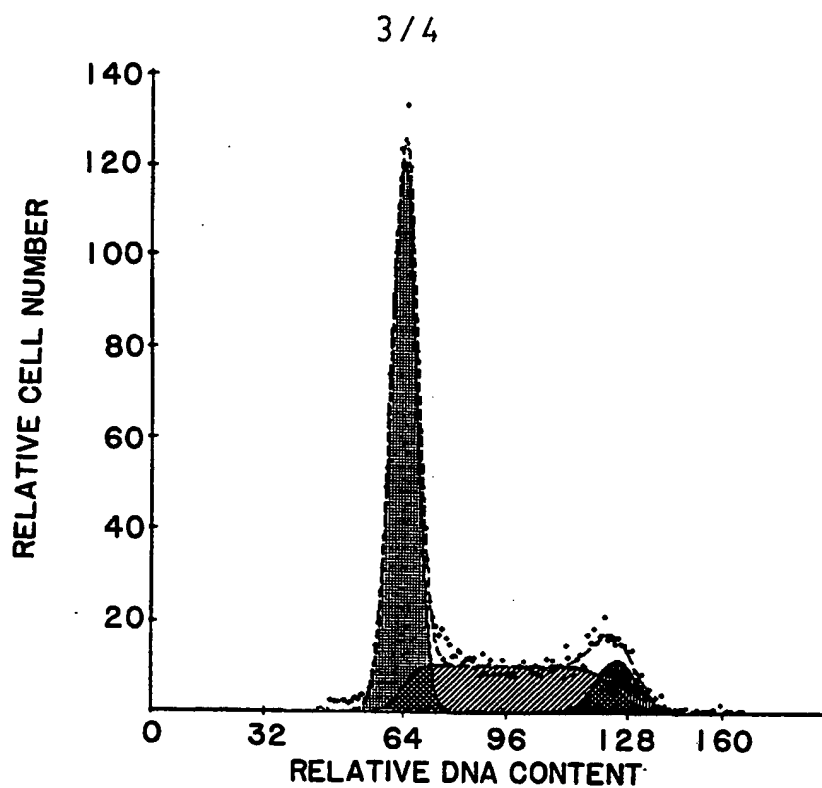
FIG. 3A
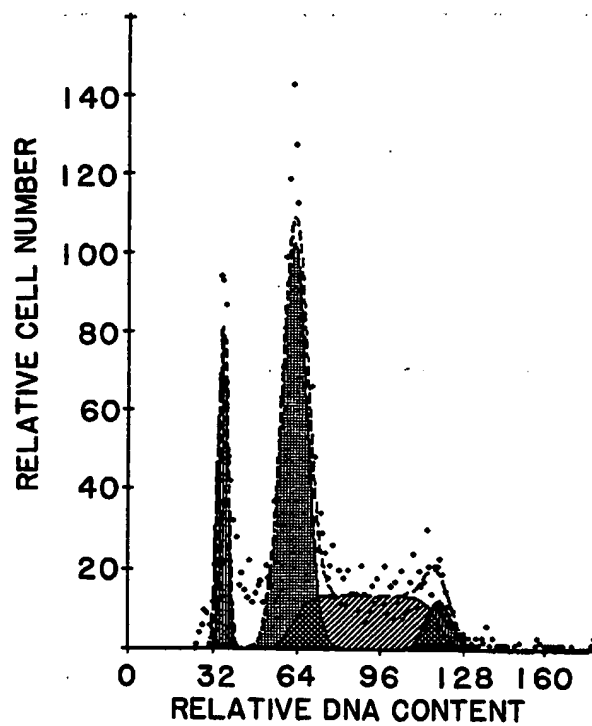
FIG. 3B   SUBSTITUTE SHEET (RULE 26)

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FIG. 3C

FIG. 3D

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 5/10, 15/87
US CL :435/240.2, 172.3

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/240.: 172.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE EXPRESS, BIOSIS, WPI
search terms: testicular, testes, germ cell

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	Biology of Reproduction, Volume 23, issued 1980, J. P. Mather, "Establishment and Characterization of Two Distinct Mouse Testicular Epithelial Cell Lines", pages 243-252, entire document.	1-4 ---- 5-31
X --- Y	Annals of the New York Academy of Sciences, Volume 383, issued 1982, J. P. Mather et al., "CULTURE OF TESTICULAR CELLS IN HORMONE-SUPPLEMENTED SERUM-FREE MEDIUM", pages 44-68, entire document.	1-4 ---- 5-31
X --- Y	Journal of Ultrastructure Research, Volume 87, issued 1984, J. P. Mather et al., "Establishment of a Peritubular Myoid-like Cell Line and Interactions between Established Testicular Cell Lines in Culture", pages 263-274, entire document.	1-4 ---- 5-31

[X] Further documents are listed in the continuation of Box C.   [ ] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
26 OCTOBER 1994	**0 3 NOV 1994**

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	Science, Volume 154, issued 02 December 1966, Y. Yasumura et al., "Establishment of Four Functional, Clonal Strains of Animal Cells in Culture", pages 1186-1189, entire document.	1-4 ---- 5-31
X --- Y	Archives of Virology, Volume 115, issued 1990, C. S. Choi et al., "Establishment of transformed swine fibroblast cell lines using SV40 large T antigen", pages 227-237, entire document.	1-9 --- 10-31
Y	Proceedings of the National Academy of Sciences (USA), Volume 75, No. 3, issued March 1978, J. Y. Chou, "Human placental cells transformed by tsA mutants of simian virus 40: A model system for the study of placental functions", pages 1409-1413, entire document.	17-22, 24-29, 31